

## The cell specificity of gene expression in the response to heat stress in corals

N. Traylor-Knowles<sup>1,2</sup>, N. H. Rose<sup>1,3</sup>, S. R. Palumbi<sup>1</sup>

<sup>1</sup> Hopkins Marine Station, Stanford University, 120 Oceanview Blvd, Pacific Grove, CA 93950, USA

<sup>2</sup> present address: University of Miami Rosenstiel School of Marine and Atmospheric Science, 4600 Rickenbacker Causeway, Florida 33149, USA

<sup>3</sup> present address: Department of Ecology and Evolutionary Biology, Princeton University, Princeton, NJ 08544, USA

Corresponding author: Nikki Traylor-Knowles, Rosenstiel School of Marine and Atmospheric Science, 4600 Rickenbacker Causeway, Florida 33149, USA,

[ntraylorknowles@rsmas.miami.edu](mailto:ntraylorknowles@rsmas.miami.edu)

## 2. ABSTRACT

Previous transcriptional studies in heat stressed corals have shown that many genes are responsive to generalized heat stress whereas the expression patterns of specific gene networks after heat stress show strong correlations with variation in bleaching outcomes. However, where these specific genes are expressed is unknown. Here we employed *in situ* hybridization to identify patterns of spatial gene expression of genes previously predicted to be involved in general stress response and bleaching. We found that Tumor Necrosis Factor Receptors (TNFRs), known to be strong responders to heat stress, were not expressed in gastrodermal symbiont-containing cells but were widely expressed in specific cells of the epidermal layer. The transcription factors AP-1 and FosB implicated as early signals of heat stress and were widely expressed throughout the oral gastrodermis and epidermis. By contrast, a G-protein coupled receptor gene (GPCR), and a fructose bisphosphate aldolase C gene (Aldolase), previously implicated in bleaching, was expressed in symbiont containing gastrodermal cells, and in epidermal tissue. Finally, Chordin-like/Kielin (Chordin-like) a gene highly correlated to bleaching was expressed solely in the oral gastrodermis. From this study we confirm that heat responsive genes occur widely in coral tissues outside of symbiont containing cells, and that gene expression in response to heat stress that causes bleaching does not signal by itself that a gene is expressed in the symbiotic cells where bleaching occurs. Joint information about expression patterns in response to heat and cell specificity will allow greater dissection of the regulatory pathways and specific cell reactions that lead to coral bleaching.

### 3. INTRODUCTION

The warming of ocean temperatures pose a threat to many ocean-dwelling organisms, especially reef-building corals, which are historically sensitive to changes in their local environment (Hoegh-Guldberg and Bruno, 2010; Hughes et al., 2007; Pandolfi et al., 2003) (Halpern et al., 2012). One of the most characterized and noticeable outcomes of these local warming events is coral bleaching. During these bleaching events, the symbiotic algae, *Symbiodinium* spp., are predominately released from the gastrodermal cells of the coral. Severe bleaching leaves corals covered by translucent tissue, allowing the white skeleton to show through, and without their algal partners, corals can become more vulnerable to other threats and stressors such as starvation and disease (Gates et al., 1992). Although a large number of studies have documented strong gene expression changes associated with bleaching conditions, little is known about whether these patterns are part of the bleaching reaction itself, or part of the heat stress response that is seen in most animals, plants and fungi (Barshis et al., 2013; Bay and Palumbi, 2015; Dixon et al., 2015; Leggat et al., 2011; Meyer et al., 2009b; Palumbi et al., 2014; Pinzon et al., 2015; Rose et al., 2015; Ruiz-Jones and Palumbi, 2014; Ruiz-Jones and Palumbi, 2015; Seneca and Palumbi, 2015). Most transcriptomics projects are based on slurries of tissue from corals, and so information concerning specificity in cell expression is lost. Additionally these studies rarely confirm their finding using cell biological techniques. As a first step, spatial gene expression visualization using histological techniques can be very revealing because it can provide visual evidence of where specific mRNAs are expressed within a cell or tissue (McFadden, 1995). Within cnidarians, this method has been primarily used in the model systems *Hydra* and *Nematostella vectensis* (Finnerty and Martindale, 1999; Kurz et al., 1991; Wolenski et al., 2013)

In addition to cell biological techniques, transcriptomic techniques can be used to identify physiological states associated with gene expression such as generalized heat responses and those involved in bleaching. Rose and colleagues took advantage of the fact that different corals individuals within a species often bleach differently even when exposed to the same standard stress, and searched for gene expression patterns predictive of bleaching results (Rose et al., 2015). They described a set of 277 genes (found in Module 12) with expression that correlated with bleaching outcomes within the coral *Acropora hyacinthus*. Additionally, Module 1 was identified as being highly enriched for a large number of general stress response-like genes, including tumor necrosis factor receptor (TNFR) genes (Rose et al., 2015). The TNFR gene family has recently been identified as having significant up-regulation in corals exposed to heat stress, but the actual function and localization of this gene response within the coral animal is still not understood (Barshis et al., 2013; Palumbi et al., 2014; Quistad and Traylor-Knowles, 2016; Quistad et al., 2014). Throughout the continuation of this manuscript, we will refer to Module 1 as “stress response module,” and Module 12 as “bleaching module.”

To begin to address the cellular specificity of heat response genes, we compared the local spatial expression patterns of stress response module and bleaching module genes in *A. hyacinthus* using *in situ* hybridization. Corals are diploblastic animals with only two cell layers. Algal symbionts (*Symbiodinium*) are contained strictly within specialized gastrodermal cells, whereas the epidermal layer has other sets of distinctive cells such as different cnidocytes including nematocytes, and spirocytes and mucous cells called mucocytes (Peters, 2016). In this study we tested the hypothesis that stress response module genes are general heat stress response genes, and would therefore be expressed by the corals cells within the

epidermis and oral gastrodermis. In parallel, we tested genes from the bleaching module with the hypothesis that expression of these genes would be restricted to symbiont-containing cells. Also, two additional genes not found in stress response module or bleaching module were tested. Using *in situ* hybridization in branches from adult corals, we found widespread expression of stress response module genes across tissue layers and more restricted expression in bleaching module genes to primarily the oral gastrodermis.

#### 4. MATERIALS AND METHODS

##### (a) *Sample preparation*

The samples used in this study, were part of two larger genomic studies on thermal tolerance (Seneca and Palumbi, 2015; Rose et al, 2016) (Supplementary Table 1). In short, small branches (approximately 25 mm in length) of *Acropora hyacinthus* were collected from six colonies and exposed to a heat stress or to control conditions as detailed in Seneca and Palumbi, 2015. Branches were sampled 5 hours from the start of the experiment (Seneca and Palumbi, 2015). Replicate branches were scored for bleaching extent (1=none, 5=total) at 20 hours. Gene expression was measured using RNASeq as in Seneca and Palumbi, 2015.

Ten heat stressed branches, and two control branches from these six colonies were preserved in 4% paraformaldehyde in filtered sea water, and then washed in phosphate-buffered saline (PBS), and stored in methanol at -20°C for later processing (Wolenski et al., 2013)(Colony information in Supplemental Table 1). Slide preparations of coral branches were prepared in RNase free conditions by IDEXX Laboratories Inc.

(Columbus, MO, USA). Samples were decalcified using Morse's solution (25% formic acid, 10% sodium citrate). Once calcium was removed, samples were processed for paraffin infiltration and embedded into tissue blocks. Prior to sectioning, microtomy equipment was cleaned and treated to be nuclease free (RNase Zap; Ambion, Inc. Houston, TX) and the water bath was filled with DEPC-treated water (Sigma, St. Louis, MO). A new microtomy knife was used to prepare 5  $\mu\text{m}$  sections from each sample. Sections were mounted onto charged microscope slides (Leica Biosystems, Inc. Buffalo Grove, IL). One section from each sample was processed for hematoxylin and eosin staining (Fischer et al., 2008).

(b) *In situ hybridization probe preparation*

The protocol for designing Anti-DIG mRNA probes was modified from Wolenski et al 2013. In short, the Digoxigenin (DIG)-labeled anti-sense, single stranded mRNA probes were designed for TNFR 4, TNFR 39, TNFR 41, AP-1, Aldolase, GPCR, Chordin-like and FosB. To begin, primers were designed to replicate amplicons approximately 400 bps in length for the genes of interest (Supplementary Table 2). Each gene was then replicated using polymerase chain reaction (PCR) and then ligated into a pGemT easy vector system (Promega, Madison, WI, USA). JM109 High Efficiency Competent Cells (Promega, Madison, WI, USA) were transformed using a 2 minute heat shock at 42°C, and diluted in 900  $\mu\text{l}$  of Super Optimal broth with Catabolite repression (SOC) medium and incubated for 60 minutes shaking 225 rpm, at 37°C. 50 $\mu\text{l}$  of each transformant was pipetted onto Lysogeny Broth (LB)/ampicillin/IPTG/X-Gal plates and grown over night at 37°C. White colonies were picked and grown overnight in 3-4 mL of

LB broth at 37°C while shaking at 150rpm. Minipreps were performed using a Qiagen Miniprep kit (Qiagen, Hilden, Germany) to purify the plasmids, and commercially available M13 primers were used in a PCR reaction for 29 cycles to further concentrate each sample. Samples were then sequenced using Sanger sequencing to verify that the correct product was cloned. Once verified using BLASTx, these products were then used to generate Anti-DIG mRNA probes with a T7 or Sp6 Megascript kit (Ambion, Thermo Fisher Scientific, Waltham, MA, USA).

(c) *In situ hybridization*

*In situ* hybridization was performed on unstained coral tissue sections following a modified protocol previously published (Ragone Calvo et al., 2001). The paraffin was dissolved with two washes of 100% xylene for 5 minutes, then gradually rehydrated using ethanol dilutions of 95%, 75% and 50%. Samples were then pre-treated in 10 mg/μl of Proteinase K in 1x phosphate-buffered saline (PBS) at 37 °C for 10 minutes. The reaction was stopped with 2% glycine in PBS wash for five minutes at room temperature. Samples were then incubated in 2x saline sodium citrate (SSC) solution for 10 minutes at room temperature.

To prepare the Anti-DIG mRNA probes for hybridization, each Anti-DIG mRNA probe was denatured while in hybridization buffer for 10 minutes at 90 °C. Tissue on each slide was enclosed using a PAP pen (Sigma Aldrich, St. Louis, MO) and 50ul of diluted Anti-DIG mRNA probe was pipetted onto the slide. Hybridization of each probe was done at 50°C overnight. The next morning slides were removed from the incubator, and washed in 2X SSC and 1X SSC, both at room temperature for 10 minutes, and 0.05X

SSC for 10 minutes at 42 °C. The slides were then washed in alkaline phosphatase (AP) buffer for 1 minute, and then incubated in Boehringer-Mannheim blocking buffer (Roche, Sigma Aldrich, St. Louis, MO) for 1 hour at room temperature. Slides were then incubated with AP-Fab fragments (Roche, Sigma Aldrich, St. Louis, MO) and Blocking buffer (Roche, Sigma Aldrich, St. Louis, MO) overnight at 4 °C. Anti-DIG mRNA probes were visualized using an alkaline phosphatase substrate, BM purple (Roche, Sigma Aldrich, St. Louis, MO) over an hour at room temperature in the dark. To stop the colorimetric reaction slides were then washed in Tris EDTA buffer for 5 minutes, then washed with water for 1 minute and mounted with glycerol. Slides were imaged using an Olympus BX43 microscope with objectives ranging from 10x–60x, and photographed using an Olympus DP21 camera attachment (Olympus, Center Valley, PA, USA).

## 5. RESULTS

### *(a) Heat stress causes severe damage to all of the coral tissue layers*

To examine the cellular architecture of coral tissues under heat stress, H&E staining was employed. The tissue and cellular architecture was drastically different between heat-stressed and control samples (Figure 1a). In the control samples, tissue epithelia and mesoglea were within normal limits. Nuclear and cytoplasmic contents were well preserved: mucocytes were scattered and not hypertrophied, with pale frothy basophilic secretion present. Spirocysts also in the epidermis stained brightly with eosin, and nematocysts were pale. Gastrodermal cells contained the symbiotic alga *Symbiodinium*, most of which were in good condition with



pink, slightly vacuolated cytoplasm, and a normal dinokaryon (nucleus), and pyrenoid body often visible. No signs of bleaching were present (Figure 1a).

In comparison, the heat-stressed samples had abnormal tissue architecture and loss of staining quality (Figure 1b). Mucocytes had increased in number in the epidermis and were lysing, as were the other vacuolated epithelial cells. The organelles of cnidocytes were present but not as well formed or stained as in the control corals; the epithelium was disrupted, releasing spirocysts and microbasic mastigophores from lysed cells. *Symbiodinium* were misshapen and fewer in number in gastrodermal cells in comparison with the control samples, with pyknotic (shrunken) or lysing nuclei, enlargement of the vacuoles in which they reside in gastrodermal cells (symbiosome), and more prominent green lipid vacuoles. Marked-to-severe necrosis and lysing of cells of both the coral and algae were present. Overall tissue staining was much lighter in the heat stressed samples, and clear delineation of the tissue layers was not as easily identified as it was in the controls. These observations confirm that our heated samples were experiencing cellular effects of heat stress, in contrast to the lack of signs of cellular heat stress in the control samples. At the time of sampling (5 hours), these corals did not look visibly paler in color, however the cellular signs of heat stress were present, and this is an important factor to consider when examining heat stressed corals (Rose et al., 2015; Seneca and Palumbi, 2015). In addition, this method confirmed that during this particular heat stress experiment, all of the tissues and cell layers in the heat stressed samples displayed signs of stress. This included not only the gastrodermal cells containing *Symbiodinium*, but also the cnidocytes and mucocytes. This finding is critical for our understanding of the heat stress response and helps to frame the gene expression work we previously conducted.

*(b) Induction of expression for genes chosen for in situ analysis:*

The coral samples we examined were part of a large study of gene expression as a function of heat stress (Rose et al., 2015; Seneca and Palumbi, 2015). In these experiments, all genes we chose for *in situ* hybridization showed induction 5 hours after heating compared to controls (Figure 2, 1.5 - 6 fold,  $p < 0.0001$ ). Stress response module genes (AP-1, FosB, and TNFR41) showed no differential expression between heavily bleached versus less bleached colonies and by 20 hours the expression had dropped substantially back towards control levels (Figure 2a). Alternatively, bleaching module genes (Chordin-like, Aldolase, and GPCR), showed marked increases of gene expression in more heavily bleached colonies, and maintained high levels of expression at the 20hr time point when bleaching became apparent (Figure 2b). By contrast, TNFR 39, and TNFR 4 increased strongly at 5 hours and returned to baseline by 20 hours (Figure 2c and 2d). Based on these results, we hypothesized that AP-1, FosB, and TNFR 41 functioned in the early response of the coral to heat stress and would be expressed widely in coral cells, but that the bleaching-sensitive Chordin-like, Aldolase, and GPCR genes might be expressed in the cells that are undergoing bleaching in the coral oral gastrodermis.

*(c) Stress response module genes: AP-1, FosB and TNFR 41*

AP-1 is a transcription factor that is activated by stress, and by other factors including cytokines and growth factors (Shaulian and Karin, 2002). This transcription factor is activated when JUN and FosB proteins dimerize (Shaulian and Karin, 2002). Additionally FosB can be active on its own, and has been linked to functions in extracellular matrix control of smooth muscle cells (Ramachandran et al., 2011). TNFR 41 is part of the Tumor Necrosis Factor

Receptor Family, which was previously found to be highly up regulated in response to heat in corals (Barshis et al., 2013; Palumbi et al., 2014; Quistad et al., 2013; Quistad and Traylor-Knowles, 2016; Quistad et al., 2014; Traylor-Knowles and Palumbi, 2014) and its role in other organisms includes activating signaling pathways leading to apoptosis or to proliferation of cells (Aggarwal, 2003; Cabal-Hierro and Lazo, 2012; Gaur and Aggarwal, 2003; MacEwan, 2002).

*In situ* localization of AP-1 and FosB was found throughout the epidermis and the oral gastrodermis, including the gastrodermal cells containing *Symbiodinium* (Figures 3a and 3b). AP-1 expression was found in spirocytes of the epidermis, while expression of FosB was found in both spirocytes and nematocytes (Figure 3a and 3b). TNFR 41 showed a slightly different pattern, where expression was primarily segregated to the cnidocytes within the cnidoglandular bands of the mesenterial filaments in the oral gastrodermis, as well as within the spirocytes and the nematocytes within the epidermis (Figure 3c).

*(d) Bleaching module genes: Chordin-like, Aldolase and GPCR*

Aldolase is an important enzyme that functions in glycolysis (Dandekar et al., 1999). GPCRs are seven transmembrane-spanning receptors that have a wide range of roles in mammals, and are one of the most abundant groups of receptors in eukaryotes (Pitcher et al., 1998). These functions include immune system regulation, and cell density sensing (Pitcher et al., 1998). Lastly Chordin-like is an important developmental gene involved in dorso-ventral patterning in vertebrates (Abreu et al., 2002; Matsui et al., 2000), as well as in renal function (Lin et al., 2005). Chordin-like was expressed only within the oral gastrodermis (Figures 5a), specifically within the gastrodermal cells containing *Symbiodinium*, and throughout the

extracellular matrix in the oral gastrodermis. In contrast, both Aldolase and GPCR were expressed in the gastrodermal cells containing *Symbiodinium*, as well as in epidermal spirocytes and nematocytes (GPCR only) (Figures 5b and 5c).

*(e) Non-responsive genes: TNFR 4 and TNFR 39*

Much like TNFR 41, the spatial gene expression of TNFR4 and TNFR 39 in heat-stressed corals included specialized cells primarily found in the epidermis, especially the nematocytes (Figures 6a and 6b). Both TNFR 4 and TNFR39 had expression within the cnidoglandular bands of the mesenterial filaments of the oral gastrodermis; however, symbiont-containing gastrodermal cells did not show localization of any TNFR gene after heat stress (Figures 4c, 6a, and 6b).

## **6. DISCUSSION**

*(a) Spatial expression of RNA is an important first step for understanding gene function*

This study utilized *in situ* hybridization to localize the expression of different genes previously shown to be responsive to heat stress in corals. These data allowed us to test whether expression included the symbiont-containing gastrodermal cells that spark the bleaching response. Expression localization shows that TNFR expression is only in the epidermal nematocytes (TNFR 4, TNFR 39 and TNFR 41), in the spirocytes (TNFR 41 only) and within the cnidoglandular bands of the mesenterial filaments in the oral gastrodermis (TNFR 4 only). Staining within symbiont-containing cells was minimal. By contrast, AP-1 and FosB both had expression throughout the epidermis and the oral gastrodermis. Lastly, Chordin-like is the most restricted to symbiont-containing cells.

Our data add to the emerging characterization that the genetic response to heat in corals is a complex mix of responses from different types of cells. Most cell types from eubacteria to single celled eukaryotes to metazoans can mount a response to an acute heat increase (Feder and Hofmann, 1999). In fact, the heat shock promoter element is one of the classic mechanisms for cells to respond to changes in the external physical environment (Feder and Hofmann, 1999). These responses are not necessarily involved in the bleaching mechanism in corals, but because they are initiated by heat, they may appear to be. For example, Seneca and Palumbi (2015) found over 5000 genes were responsive to heat stress in corals, however, expression in only about 10% of these differed in corals that bleached after heating compared to conspecifics that did not (Rose et al., 2015).

Because coral gene expression studies derive from mixtures of all cell types and tissue layers, prior studies of the heat response have been unable to dissect the different cellular roles of different genes. Our initial *in situ* hybridization data suggest that there is a set of genes that are localized to the symbiont-containing cells. Some of these are also expressed in epidermal tissues, but one of our target genes, Chordin-like is only upregulated in symbiont-containing cells.

*(b) The role of TNFRs in heat stress*

Transcriptomic evidence shows that multiple TNFR genes are activated quickly during heat stress, and expressed in a coordinated fashion (Barshis et al., 2013; Palumbi et al., 2014; Seneca and Palumbi, 2015). The evidence we report here suggests that these TNFRs, and by extension perhaps more genes of the stress response module (Module 1) from Rose *et al.* (2015), are not directly involved in the bleaching mechanism but rather are part of the

generalized stress response (Rose et al., 2015). The broad range of TNFR types, and signaling networks they can activate, make them likely candidates for roles in stress modulation (Traylor-Knowles and Palumbi, 2014). Untangling the TNFR signaling mechanisms in corals will be challenging. There are many different types (42 total) and they possess rich sequence diversity (Quistad and Traylor-Knowles, 2016).

*(c) AP-1 and FosB as heat stress regulators*

The AP-1 transcription factor is an important transcriptional regulator for general stress response in many organisms including humans and mice (Shaulian and Karin, 2002). In this study, we showed that both AP-1 and its subunit FosB are both expressed within the symbiont-containing cells as well as within spirocytes and nematocytes (FosB only), suggesting that these genes are important in overall stress response in corals to heat stress (Figures 4a and 4b). Despite not having membership in the bleaching module, expression of these genes within the symbiont-containing cells also indicates that they could have a role in bleaching or symbiosis, but this would need to be further explored.

*(d) The role of Aldolase, Chordin-like, and GPCR in the mechanism of bleaching*

The fundamental mechanisms of bleaching are still not well understood. Recently, Bieri and colleagues showed that of all proposed types of bleaching mechanisms during heat stress, exocytosis of the symbiont cell was the most prevalent (Bieri et al., 2016). Both GPCRs and Aldolase are implicated in exocytosis of insulin through the insulin pathway (Kao et al., 1999; Madiraju and Poitout, 2007). In the insulin pathway, GPCRs act as modulators of insulin secretion, and Aldolase acts as a scaffolding protein for the glutamate receptor, which

regulates glucose transport (Kao et al., 1999; Madiraju and Poitout, 2007). In addition, glucose has been reported as the dominant metabolite transferred between *Symbiodinium* and the coral host cell (Burriesci et al., 2012). Glucose phosphorylation and the GPCR system are needed for the synthesis of cAMP (Rolland et al., 2001), an important component in cell proliferation of symbiodinium (Wang et al., 2008). Further studies will need to be done to elucidate the functional role of these genes, but these expression data is a first step in pinpointing the possible mechanisms of coral bleaching. In addition to this, the expression of these genes is found within the spirocytes and in some cases the nematocytes could also indicate that these genes are involved in other stress related processes and important to the overall health of the coral.

Chordin-like had very specific staining to the oral gastrodermis, and there was no staining found within the epidermis (Figure 5a). In mammalian models, Chordin-like acts to increase bone morphogenic protein signaling (BMP) in a paracrine fashion thus influencing renal health in adults and dorso-ventral patterning during development (Abreu et al., 2002; Lin et al., 2005; Matsui et al., 2000). However, the role of this gene in corals is not understood. The expression of this gene within the oral gastrodermis and its previously found high correlation to the bleaching phenotype (Rose et al., 2015) leads us believe that this gene may have a role in the bleaching response, but further investigation would need to be done to verify this.

*(e) The role of cnidocytes in response to heat stress*

One of the most surprising findings of this study was that the cnidocytes, specifically spirocytes and nematocytes were expressing genes involved in stress response and bleaching. Spirocytes and nematocytes are a subgroup of specialized cells called cnidocytes, which are a characteristic cell type of cnidarians (Mariscal, 1984). Spirocytes are primarily activated by the stimulus of food, and create a fibrous-like matrix on discharge of their solid tubules to increase surface area and the stickiness of the tentacle (Mariscal, 1984; Mariscal et al., 1976; Mariscal and McLean, 1976; Westfall et al., 1999), whereas nematocytes are stinging cells used during prey-capture and inter-colony aggression (Hidaka, 1985). While, further investigations on these cell types needs to be done, it is possible that they play an important role in generalized stress response.

Previous studies in the starlet sea anemone, *Nematostella vectensis*, have found that nematostomes, structures that contain cnidocytes, as well as other cell types, are part of the *Nematostella vectensis* immune system (Babonis et al. 2016). Also, Wolenski and co-authors found that NF- $\kappa$ B, a generalized stress response transcription factor, was required for cnidocyte development, and expressed in specific cnidocyte populations in adult anemones (Wolenski et al., 2013). Together this evidence points to novel functions of these cells types and highlights that future investigations of cnidocytes, particularly in corals, is needed to truly understand their complex diversity and function.



*(f) Conclusions*

Our study presents evidence that genes previously found to be correlated with stress response and bleaching response are spatially localized in different coral cell types. This expression of particular genes in the areas surrounding the symbiont suggest that the bleaching response and the stress response may be two different, but interacting mechanisms important to the coral's response to changes in their environment.

Using *in situ* hybridization techniques to localize expression patterns is a first step towards separating the roles of different genes in coral bleaching. Utilizing this technique will help researchers have a better understanding of the functional significance of genes found in large-scale sequencing projects and will allow for better biomarker development. However, there are other tools that are being developed that show promise in more fine-scale dissection of cellular roles of genes in bleaching. For example, in the invertebrate *Botryllus schlosseri*, the use of FACS and RNAseq has enabled researchers to discover the cell types that are the vertebrate precursor of the hematopoietic stem cell (Rosental et al., 2016). This single cell or cell population analysis allows for fine scale resolution of the stress response, with the focus on cellular interactions, rather than organismal reaction. Application of such tools to coral cells may provide a critical boost to further disentangling the general heat response from the specific bleaching response in these animals.

## 7. LIST OF SYMBOLS AND ABBREVIATIONS

BMP: bone morphogenic protein signaling, GPCR: G-protein coupled receptor gene, Aldolase: fructose biphosphate aldolase C gene, Chordin-like: Chordin-like/Kielin, TNFR: tumor necrosis factor receptor, Anti-DIG: Digoxigenin-labeled anti-sense, PCR: polymerase chain reaction, SOC: Super Optimal broth with Catabolite repression medium, LB: Lysogeny Broth, PBS: phosphate-buffered saline, SSC: saline sodium citrate solution

## 8. APPENDIX

**Supplementary Table 1:** Information on the samples that we used in this study including symbiont type and treatment.

**Supplementary Table 2:** Primers used in this study to produce in situ hybridization probes.

## 9. ACKNOWLEDGEMENTS

The authors would like to thank Dr. Esther Peters of George Mason University for assistance with slide reading and for reading the first drafts of this paper. Her comments were incredibly valuable. The authors would also like to thank Dr. Abby Renegar for access to the microscopes of the NOVA University histopathology laboratory. Lastly, the authors would like to thank all of the reviewers for their valuable comments and suggestions.

## 10. COMPETING INTERESTS

No competing interests declared.

## **11. AUTHOR CONTRIBUTIONS**

Conceptualization: N.T.K., N.H.R., S.R.P.; Methodology: N.T.K., N.H.R.; Formal analysis and investigation: N.T.K., N.H.R., S.R.P.; Writing - original draft preparation: N.T.K., S.R.P.; Writing - review and editing: N.T.K., N.H.R., S.R.P.; Funding acquisition: N.T.K., S.R.P.; Resources: N.T.K., S.R.P.; Supervision: S.R.P.

## **12. FUNDING**

NTK was funded by: Award # OCE-1323652 through the National Science Foundation Ocean Science Postdoctoral Fellowship and award #1012629 from the Burroughs Wellcome Postdoctoral Enrichment Fund. SRP was funded by grant # GBMF2629 of the Gordon and Betty Moore Foundation. NHR was funded by National Science Foundation Graduate Research Fellowship.

### 13. REFERENCES:

- Abreu, J., Coffinier, C., Larrain, J., Oelgeschläger, M. and De Robertis, E.** (2002). Chordin-like CR domains and the regulation of evolutionarily conserved extracellular signaling systems. *Gene* **287**, 39-47.
- Aggarwal, B. B.** (2003). Signalling pathways of the TNF superfamily: A double-edged sword. *Nature Reviews Immunology* **3**, 745-756.
- Babonis, L.S., Martindale, M.Q., Ryan, J.F.** (2016) Do novel genes drive morphological novelty? An investigation of the nematostomes in the sea anemone *Nematostella vectensis*. *BMC Evolutionary Biology* **16**.
- Barshis, D. J., Ladner, J. T., Oliver, T. A., Seneca, F. O., Traylor-Knowles, N. and Palumbi, S. R.** (2013). A genomic basis for coral resilience to climate change. *Proc Natl Acad Sci U S A.* **10.1073/pnas.1210224110**
- Bay, R. A. and Palumbi, S. R.** (2015). Rapid Acclimation Ability Mediated by Transcriptome Changes in Reef-Building Corals. *Genome Biology and Evolution* **7**, 1602-1612.
- Bieri, T., Onishi, M., Xiang, T. T., Grossman, A. R. and Pringle, J. R.** (2016). Relative contributions of various cellular mechanisms to loss of algae during cnidarian bleaching. *PLoS One* **11**.
- Burriesci, M. S., Raab, T. K. and Pringle, J. R.** (2012). Evidence that glucose is the major transferred metabolite in dinoflagellate-cnidarian symbiosis. *Journal of Experimental Biology* **215**, 3467-3477.
- Cabal-Hierro, L. and Lazo, P. S.** (2012). Signal transduction by tumor necrosis factor receptors. *Cellular Signalling* **24**, 1297-1305.
- Dandekar, T., Schuster, S., Snel, B., Huynen, M. and Bork, P.** (1999). Pathway alignment: application to the comparative analysis of glycolytic enzymes. *Biochemical Journal* **343**, 115-124.
- DeSalvo, M. K., Sunagawa, S., Voolstra, C. R. and Medina, M.** (2010). Transcriptomic responses to heat stress and bleaching in the elkhorn coral *Acropora palmata*. *Marine Ecology Progress Series* **402**, 97-113.
- Dixon, G. B., Davies, S. W., Aglyamova, G. A., Meyer, E., Bay, L. K. and Matz, M. V.** (2015). Genomic determinants of coral heat tolerance across latitudes. *Science* **348**, 1460-1462.
- Feder, M. E. and Hofmann, G. E.** (1999). Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. *Annual Review of Physiology* **61**, 243-282.
- Finnerty, J. R. and Martindale, M. Q.** (1999). Ancient origins of axial patterning genes: Hox genes and ParaHox genes in the Cnidaria. *Evol Dev* **1**, 16-23.
- Fischer, A. H., Jacobson, K. A., Rose, J. and Zeller, R.** (2008). Hematoxylin and eosin staining of tissue and cell sections. *CSH Protoc* **2008**, pdb prot4986.
- Gates, R. D., Baghdasarian, G. and Muscatine, L.** (1992). Temperature Stress Causes Host-Cell Detachment in Symbiotic Cnidarians - Implications for Coral Bleaching. *Biological Bulletin* **182**, 324-332.

- Gaur, U. and Aggarwal, B. B.** (2003). Regulation of proliferation, survival and apoptosis by members of the TNF superfamily. *Biochem Pharmacol* **66**, 1403-8.
- Halpern, B. S., Longo, C., Hardy, D., McLeod, K. L., Samhoury, J. F., Katona, S. K., Kleisner, K., Lester, S. E., O'Leary, J., Ranelletti, M. et al.** (2012). An index to assess the health and benefits of the global ocean. *Nature* **488**, 615-20.
- Hidaka, M.** (1985). Nematocyst discharge, histoincompatibility, and the formation of sweeper tentacles in the coral *galaxea fascicularis*. *Biological Bulletin* **168**, 350-358.
- Hoegh-Guldberg, O. and Bruno, J. F.** (2010). The impact of climate change on the world's marine ecosystems. *Science* **328**, 1523-8.
- Hughes, T. P., Rodrigues, M. J., Bellwood, D. R., Ceccarelli, D., Hoegh-Guldberg, O., McCook, L. J., Moltschaniwskyj, N., Pratchett, M., Steneck, R. S. and Willis, B.** (2007). Phase shifts, herbivory, and the resilience of coral reefs to climate change. *Current Biology* **17**, 360-365.
- Kao, A. W., Noda, Y., Johnson, J. H., Pessin, J. E. and Saltiel, A. R.** (1999). Aldolase mediates the association of F-actin with the insulin-responsive glucose transporter GLUT4. *Journal of Biological Chemistry* **274**, 17742-17747.
- Kenkel, C. D., Meyer, E. and Matz, M. V.** (2013). Gene expression under chronic heat stress in populations of the mustard hill coral (*Porites astreoides*) from different thermal environments. *Molecular Ecology* **22**, 4322-4334.
- Kurz, E. M., Holstein, T. W., Petri, B. M., Engel, J. and David, C. N.** (1991). Mini-Collagens in Hydra Nematocytes. *Journal of Cell Biology* **115**, 1159-1169.
- Leggat, W., Seneca, F., Wasmund, K., Ukani, L., Yellowlees, D. and Ainsworth, T. D.** (2011). Differential Responses of the Coral Host and Their Algal Symbiont to Thermal Stress. *PLoS One* **6**.
- Lin, J. M., Patel, S. R., Cheng, X., Cho, E. A., Levitan, I., Ullenbruch, M., Phan, S. H., Park, J. M. and Dressler, G. R.** (2005). Kielin/chordin-like protein, a novel enhancer of BMP signaling, attenuates renal fibrotic disease. *Nature Medicine* **11**, 387-393.
- MacEwan, D. J.** (2002). TNF ligands and receptors - a matter of life and death. *British Journal of Pharmacology* **135**, 855-875.
- Madiraju, S. R. M. and Poitout, V.** (2007). G protein-coupled receptors and insulin secretion: 119 and counting. *Endocrinology* **148**, 2598-2600.
- Mariscal, R. N.** (1984). Cnidaria: Cnidae. In *Biology of Integument*, pp. 57-68. Berlin, Heidelberg: Springer-Verlag.
- Mariscal, R. N., Bigger, C. H. and McLean, R. B.** (1976). The form and function of cnidarian spirocysts. 1. Ultrastructure of the capsule exterior and relationship to the tentacle sensory surface. *Cell Tissue Res* **168**, 465-74.
- Mariscal, R. N. and McLean, R. B.** (1976). The form and function of cnidarian spirocysts. 2. Ultrastructure of the capsule tip and wall and mechanism of discharge. *Cell Tissue Res* **169**, 313-21.
- Matsui, M., Mizuseki, K., Nakatani, J., Nakanishi, S. and Sasai, Y.** (2000). Xenopus kielin: A dorsalizing factor containing multiple chordin-type repeats secreted from the embryonic midline. *Proc Natl Acad Sci U S A* **97**, 5291-6.
- McFadden, G. I.** (1995). In situ hybridization. In *Methods in Cell Biology*, vol. 49, pp. 165-283: Academic Press.

- Meyer, E., Aglyamova, G. V., Wang, S., Buchanan-Carter, J., Abrego, D., Colbourne, J. K., Willis, B. L. and Matz, M. V.** (2009a). Sequencing and de novo analysis of a coral larval transcriptome using 454 GSFlx. *BMC Genomics* **10**.
- Meyer, E., Aglyamova, G. V., Wang, S., Buchanan-Carter, J., Abrego, D., Colbourne, J. K., Willis, B. L. and Matz, M. V.** (2009b). Sequencing and de novo analysis of a coral larval transcriptome using 454 GSFlx. *BMC Genomics* **10**, 219.
- Palumbi, S. R., Barshis, D. J., Traylor-Knowles, N. and Bay, R. A.** (2014). Mechanisms of reef coral resistance to future climate change. *Science* **344**, 895-898.
- Pandolfi, J. M., Bradbury, R. H., Sala, E., Hughes, T. P., Bjorndal, K. A., Cooke, R. G., McArdle, D., McClenachan, L., Newman, M. J., Paredes, G. et al.** (2003). Global trajectories of the long-term decline of coral reef ecosystems. *Science* **301**, 955-8.
- Peters, E. C.** (2016). Diseases of corals. In *Diseases of corals*, eds. C. M. Woodley C. A. Downs A. W. Bruckner J. W. Porter and S. B. Galloway), pp. 85-107. Hoboken, New Jersey, USA: John Wiley and Sons, Inc.
- Pinzon, J. H., Kamel, B., Burge, C. A., Harvell, C. D., Medina, M., Weil, E. and Mydlarz, L. D.** (2015). Whole transcriptome analysis reveals changes in expression of immune-related genes during and after bleaching in a reef-building coral. *R Soc Open Sci* **2**, 140214.
- Pitcher, J. A., Freedman, N. J. and Lefkowitz, R. J.** (1998). G protein-coupled receptor kinases. *Annual Review of Biochemistry* **67**, 653-692.
- Portune, K. J., Voolstra, C. R., Medina, M. and Szmant, A. M.** (2010). Development and heat stress-induced transcriptomic changes during embryogenesis of the scleractinian coral *Acropora palmata*. *Marine Genomics* **3**, 51-62.
- Quistad, S., Stotland, A., Barott, K., Grasis, J., Wolkowicz, R. and Rohwer, F.** (2013). The evolution of TNF-induced apoptosis reveals 550 million years of functional conservation. *Journal of Immunology* **190**.
- Quistad, S. and Traylor-Knowles, N.** (2016). Precambrian origins of the TNFR superfamily. *Cell Death Discovery* **2**.
- Quistad, S. D., Stotland, A., Barott, K. L., Smurthwaite, C. A., Hilton, B. J., Grasis, J. A., Wolkowicz, R. and Rohwer, F. L.** (2014). Evolution of TNF-induced apoptosis reveals 550 My of functional conservation. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 9567-9572.
- Ragone Calvo, L. M., Stokes, N. A. and Burreson, E. M.** (2001). Molecular methods for the detection of Quahog parasite unknown (QPX). In *Virginia Sea Grant Marine Advisory Program*.
- Ramachandran, A., Gong, E. M., Pelton, K., Ranpura, S. A., Mulone, M., Seth, A., Gomez, P., 3rd and Adam, R. M.** (2011). FosB regulates stretch-induced expression of extracellular matrix proteins in smooth muscle. *Am J Pathol* **179**, 2977-89.
- Rodriguez-Lanetty, M., Harii, S. and Hoegh-Guldberg, O.** (2009). Early molecular responses of coral larvae to hyperthermal stress. *Molecular Ecology* **18**, 5101-5114.
- Rolland, F., Winderickx, J. and Thevelein, J. M.** (2001). Glucose-sensing mechanisms in eukaryotic cells. *Trends in biochemical sciences* **26**, 310-317.

**Rose, N. H., Seneca, F. O. and Palumbi, S. R.** (2015). Gene networks in the wild: identifying transcriptional modules that mediate coral resistance to experimental heat stress. *Genome Biol Evol.*

**Rosental, B., Kowarsky, M. A., Corey, D. M., Ishizuka, K. J., Palmeri, K. J., Chen, S. Y., Sinha, R., Voskoboynik, A. and Weissman, I. L.** (2016). Finding the evolutionary precursor of vertebrate hematopoietic lineage: Functional and molecular characterization of *B. schlosseri* immune system. *Journal of Immunology* **196**.

**Ruiz-Jones, G. J. and Palumbi, S. R.** (2014). Coral transcriptome-wide gene expression patterns at noon and midnight in a coral reef: insights into coral physiology. *Integrative and Comparative Biology* **54**, E179-E179.

**Ruiz-Jones, L. J. and Palumbi, S. R.** (2015). Transcriptome-wide Changes in Coral Gene Expression at Noon and Midnight Under Field Conditions. *Biol Bull* **228**, 227-41.

**Seneca, F. O. and Palumbi, S. R.** (2015). The role of transcriptome resilience in resistance of corals to bleaching. *Mol Ecol* **24**, 1467-84.

**Shaulian, E. and Karin, M.** (2002). AP-1 as a regulator of cell life and death. *Nature Cell Biology* **4**, E131-E136.

**Traylor-Knowles, N. and Palumbi, S. R.** (2014). Translational environmental biology: cell biology informing conservation. *Trends Cell Biol* **24**, 265-7.

**Voolstra, C. R., Schnetzer, J., Peshkin, L., Randall, C. J., Szmant, A. M. and Medina, M.** (2009). Effects of temperature on gene expression in embryos of the coral *Montastraea faveolata*. *BMC Genomics* **10**.

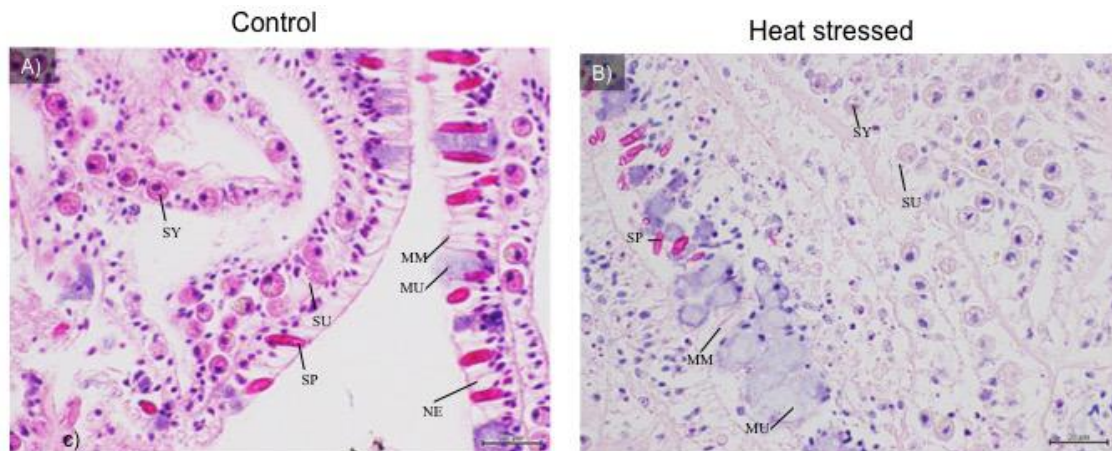
**Wang, L. H., Liu, Y. H., Ju, Y. M., Hsiao, Y. Y., Fang, L. S. and Chen, C. S.** (2008). Cell cycle propagation is driven by light-dark stimulation in a cultured symbiotic dinoflagellate isolated from corals. *Coral Reefs* **27**, 823-835.

**Westfall, J. A., Landers, D. D. and McCallum, J. D.** (1999). Ultrastructure of neuro-spirocyte synapses in the sea anemone *Aiptasia pallida* (Cnidaria, Anthozoa, Zoantharia). *J Morphol* **241**, 165-73.

**Wolenski, F. S., Layden, M. J., Martindale, M. Q., Gilmore, T. D. and Finnerty, J. R.** (2013). Characterizing the spatiotemporal expression of RNAs and proteins in the starlet sea anemone, *Nematostella vectensis*. *Nature Protocols* **8**, 900-915.

**Wolenski, F.S., Bradhman, C.A., Finnerty, J.R., Gilmore, T.D.** (2013) NF-kB is required for cnidocyte development in *Nematostella vectensis*. *Dev. Biol.* **373**, 205-2015.

## Figures

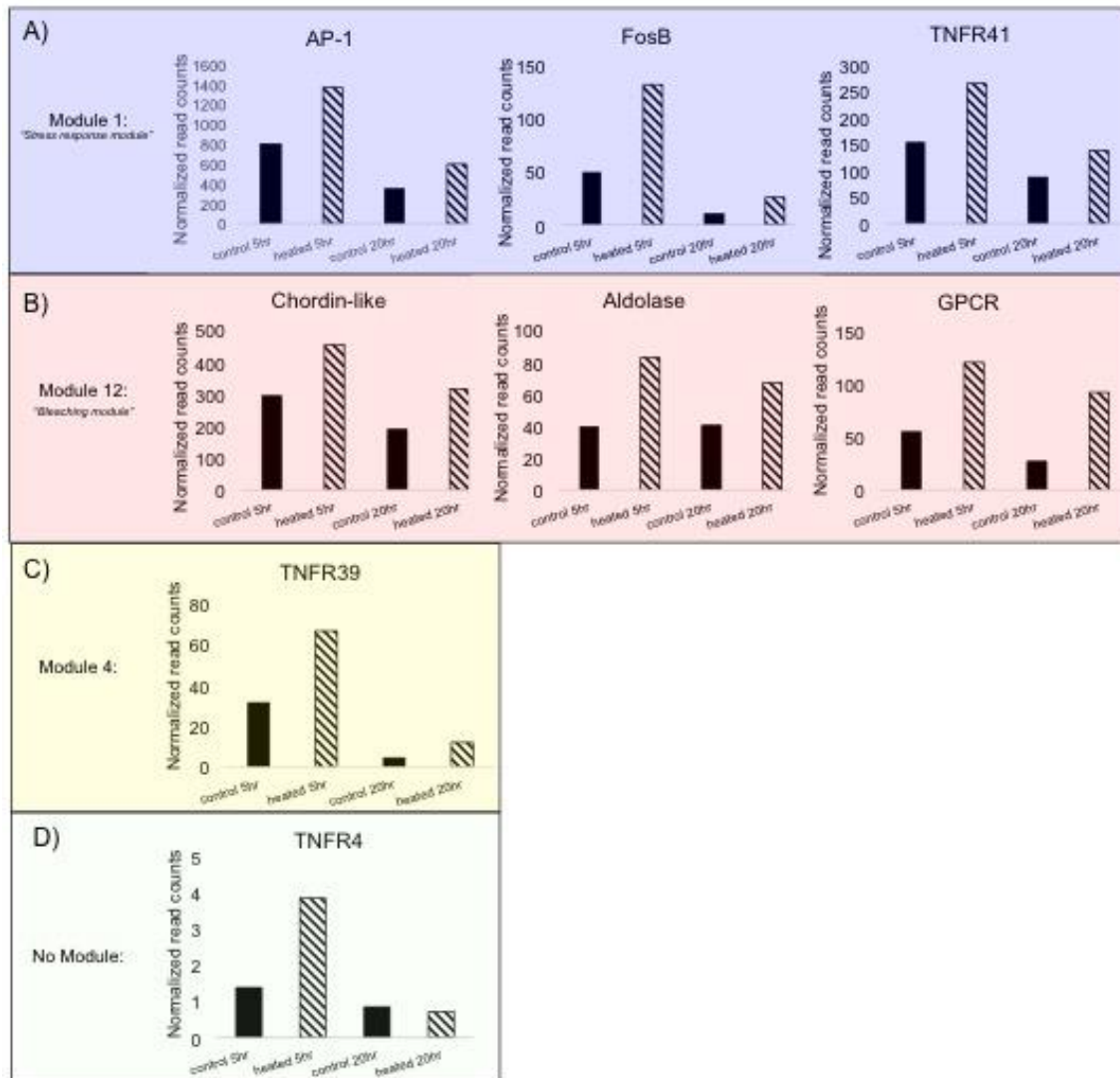


**Figure 1. H&E stained histological cross-sections of control and heat stress *A. hyacinthus*.**

Abbreviations SY: Symbiodinium, SP: Spirocyte, SU: symbiont containing gastrodermal cell, MM: microbasic mastigophores, MU: mucocytes, NE: nematocytes. (a) In the control samples tissue epithelia and mesoglea were intact and normal, with well preserved nuclear and cytoplasmic contents. Mucocytes were scattered and not hypertrophied. Spirocysts also in the epidermis stained brightly with eosin while nematocysts were pale. Gastrodermal cells contained the symbiotic algae *Symbiodinium*, which was in good condition with pink, slightly vacuolated cytoplasm, and a normal nucleus, and pyrenoid body often visible. No signs of bleaching were present. (b) Heat-stressed samples had abnormal tissue architecture and loss of staining quality. Within the epidermis, mucocytes increased and both vacuolated epithelial cells and mucocytes lysed. Cnidocytes were present but were misshaped or stained weakly.

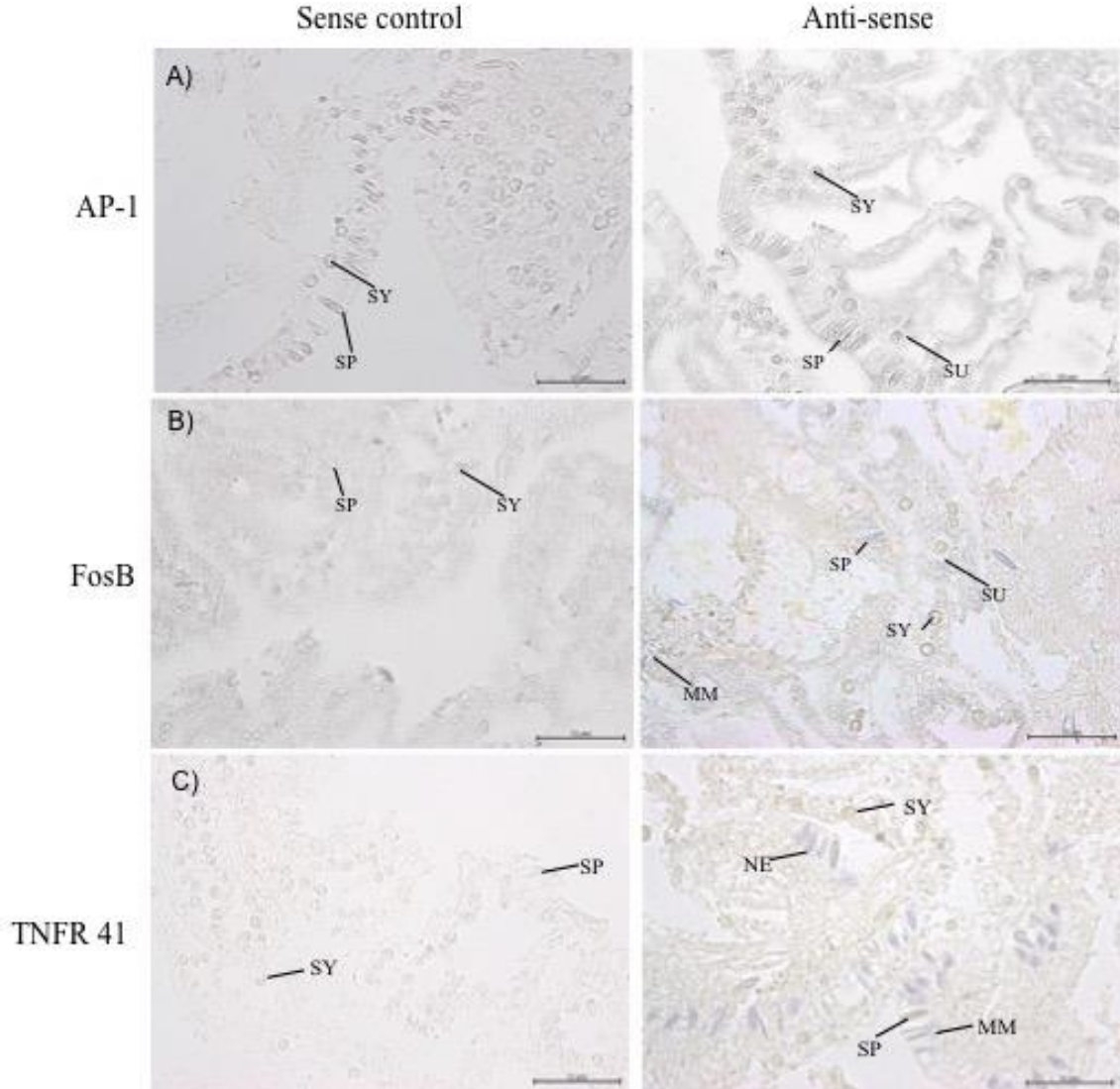


Additionally, epithelium was disrupted, releasing spirocysts and microbasic mastigophores from lysed cells. *Symbiodinium* were irregularly shaped, fewer in number and possessed shrunken or lysed nuclei with enlargement of the vacuoles in which they reside in gastrodermal cells (symbiosome), and more prominent green lipid vacuoles.



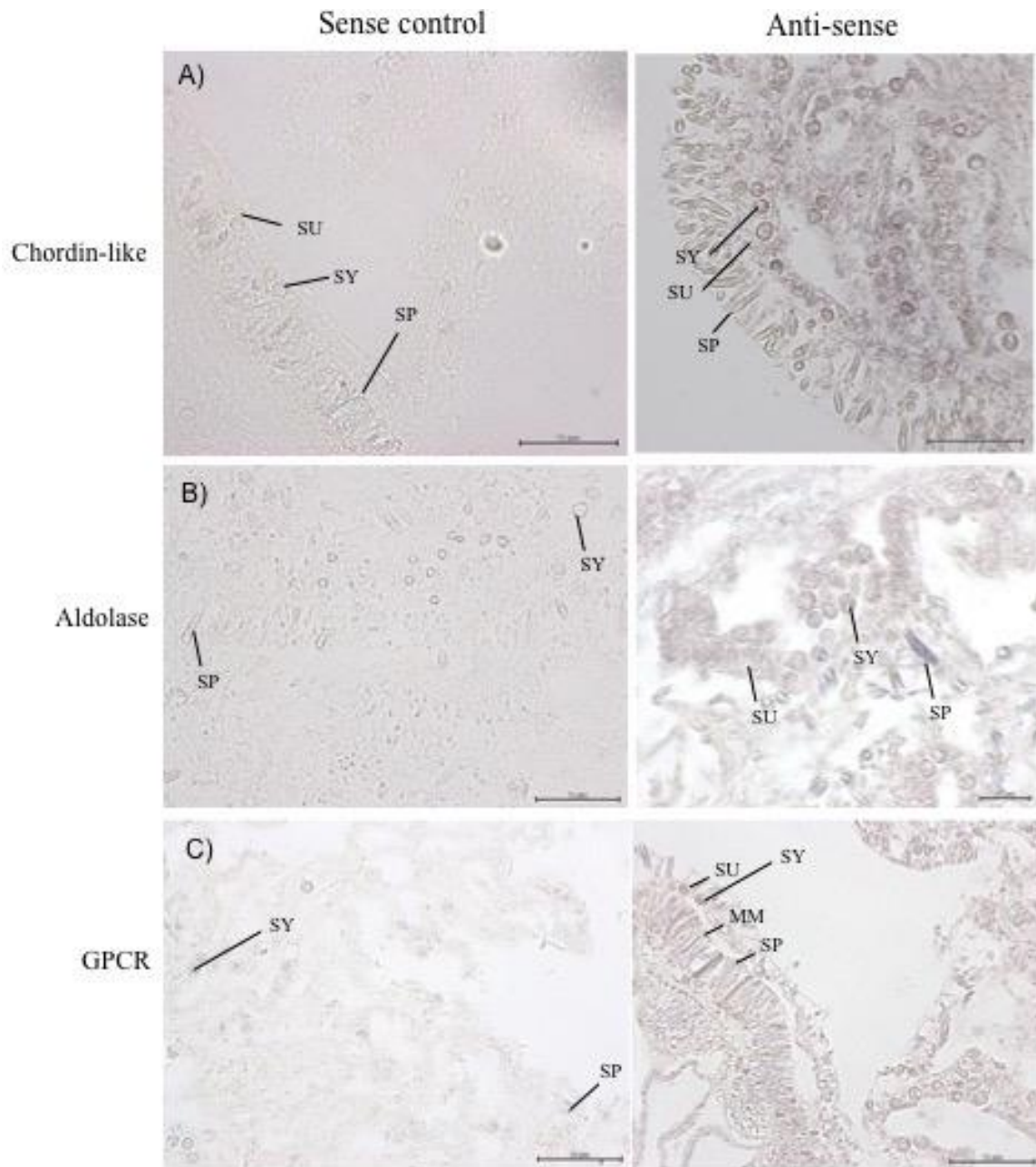
**Figure 2. Gene expression of stress response module genes (AP-1, FosB, TNFR 41), bleaching module genes (Chordin-like, Aldolase, GPCR), Module 4 (TNFR 39) and No Module (TNFR 4).** The coral samples in this study were previously part of a large study on the gene expression induced by heat stress (Rose et al., 2015; Seneca and Palumbi, 2015). (a) Stress response module genes (AP-1, FosB, and TNFR41) showed no differential expression between heavily bleached versus less bleached colonies and by 20 hours the expression had

dropped substantially back towards control levels. (b) Bleaching module genes (Chordin-like, Aldolase, and GPCR), showed gene expression induction 5 hours after heating with marked increases of gene expression in more heavily bleached colonies, and maintained high levels of expression at the 20hr time point when bleaching became apparent. (c and d) TNFR 39 part of Module 4, and TNFR 4 part of no module, increased strongly at 5 hours and returned to baseline by 20 hours.



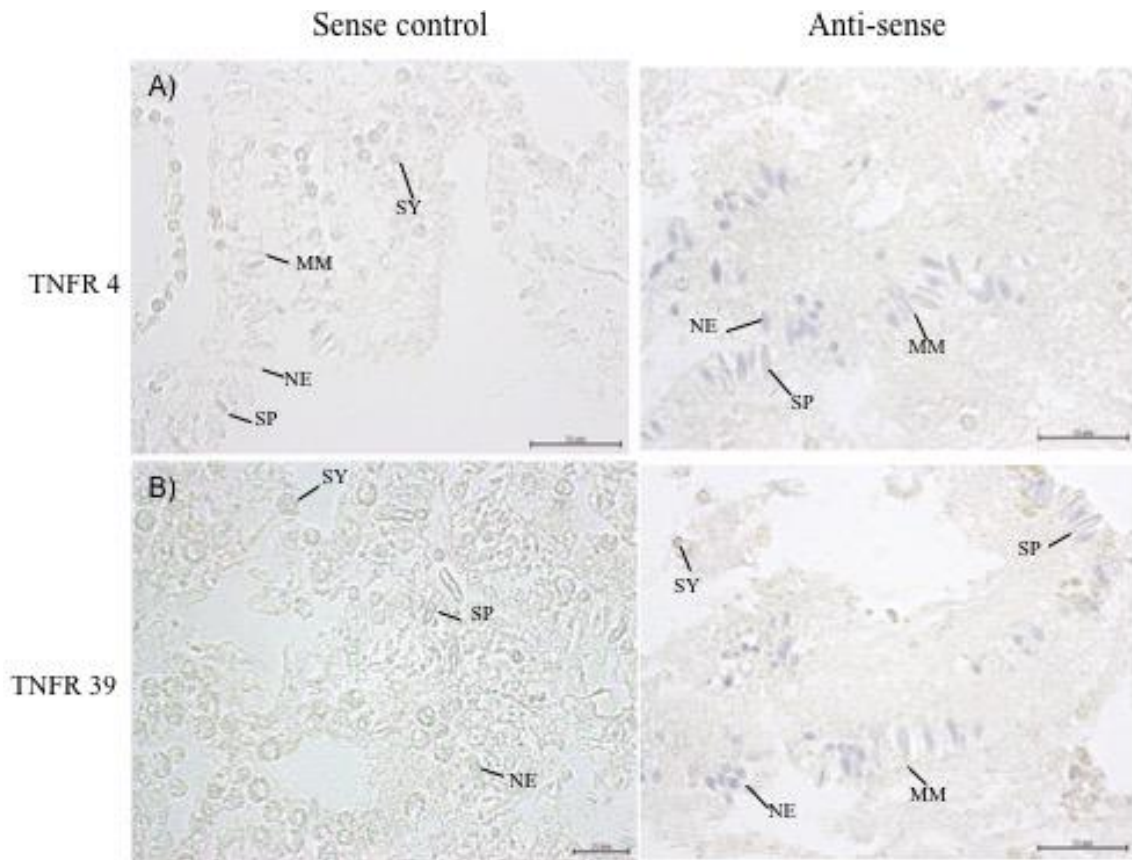
**Figure 3. Spatial expression of stress response module genes.** Abbreviations SY: Symbiodinium, SP: Spirocyte, SU: symbiont containing gastrodermal cell, MM: microbasic mastigophores, MU: mucocytes, NE: nematocytes. (a) AP-1 expression is found throughout the epidermis and the oral gastrodermis, including the gastrodermal cells containing *Symbiodinium* as well as within the in spirocytes of the epidermis. (b) FosB expression is also found throughout the epidermis and the oral gastrodermis, including the gastrodermal cells

containing *Symbiodinium* as well as in both spirocytes and nematocytes. (c) TNFR 41 expression was primarily segregated to the cnidocytes within the cnidoglandular bands of the mesenterial filaments in the oral gastrodermis, as well as within the spirocytes and the nematocytes within the epidermis.



**Figure 4. Spatial expression of bleaching module genes.** Abbreviations SY: Symbiodinium, SP: Spirocyte, SU: symbiont containing gastrodermal cell, MM: microbasic mastigophores, MU: mucocytes, NE: nematocytes. (a) Chordin-like was expressed only within the oral

gastrodermis specifically within the gastrodermal cells containing *Symbiodinium*, and throughout the extracellular matrix of the oral gastrodermis. (b) Aldolase was expressed in the gastrodermal cells containing *Symbiodinium*, as well as in epidermal spirocytes. (c) GPCR was also expressed in the symbiont containing gastrodermal cells, as well as in both nematocytes and spirocytes.



**Figure 5. Spatial expression of TNFR 39 (Module 4) and TNFR 4 (no assigned module).**

Abbreviations SY: Symbiodinium, SP: Spirocyte, SU: symbiont containing gastrodermal cell, MM: microbasic mastigophores, MU: mucocytes, NE: nematocytes. (a) TNFR 4 was expressed primarily in the nematocytes and within the cnidoglandular bands of the mesenterial filaments of the oral gastrodermis. (b) TNFR 39 was also expressed primarily in the nematocytes and with the cnidoglandular bands of the mesenterial filaments of the oral gastrodermis.



## Tables

**Table 1: Summary of target genes and expression patterns**

Gene Name	Contig Number	Uniprot ID	Rose et al, 2015 module number	Module Phenotype correlation from Rose et al, 2015	Tissue	Cell
AP-1	contig211469_14890 1_120483	P05627	1	Stress response	Oral gastrodermis, Epidermis	Gastrodermal cells, Spirocytes
FosB	contig182044	P13346	1	Stress response	Oral gastrodermis, Epidermis	Gastrodermal cells, Spirocytes, Nematocytes
TNFR41	contig151747	P08138	1	Stress response	Cnidoglandular band (Oral gastrodermis)	Nematocytes, Spirocytes
Chordin-like	contig78169	Q9IBG7	12	Bleaching	Oral gastrodermis	Gastrodermal cells
Fructose-bisphosphate aldolase C	contig108694	P53448	12	Bleaching	Oral gastrodermis Epidermis	Gastrodermal cells, Spirocytes
GPCR	contig77727	Q8IZP9	12	Bleaching	Oral gastrodermis, Epidermis	Gastrodermal cells, Nematocytes, Spirocytes
TNFR4	contig82602	P47741	n/a	n/a	Cnidoglandular band (Oral gastrodermis)	Nematocytes
TNFR39	contig151755	P08138	4	n/a	Cnidoglandular band (Oral gastrodermis)	Nematocytes

**Table S1:** Information on the samples that we used in this study including symbiont type and treatment.

[Click here to Download Table S1](#)

**Table S2:** Primers used in this study to produce in situ hybridization probes.

[Click here to Download Table S2](#)